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# **The Microbial Database for Danish wastewater treatment plants with nutrient removal (MiDas-DK) – a tool for understanding activated sludge population dynamics and community stability**

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## **Abstract**

Since 2006 more than 50 Danish full-scale WWTP with nutrient removal has been investigated in a project called “The Microbial Database for Danish Activated Sludge Wastewater Treatment Plants with Nutrient Removal (MiDas-DK)”. Comprehensive sets of samples have been collected, analyzed and associated with extensive operational data from the plants. The community composition was analyzed by quantitative FISH supported by 16S rRNA amplicon sequencing and deep metagenomics. MiDas-DK has been a powerful tool to study the complex activated sludge ecosystems, and besides many scientific articles on fundamental issues on mixed communities encompassing nitrifiers, denitrifiers, bacteria involved in P-removal, hydrolysis, fermentation, and foaming, the project has provided results that can be used to optimize the operation of full-scale plants and carry out trouble-shooting. A core microbial community has been defined comprising the majority of microorganisms present in the plants. Time series have been established, providing an overview of temporal variations in the different plants. Interestingly, although most microorganism were present in all plants, there seemed to be plant-specific factors that controlled the population composition thereby keeping it unique in each plant over time. Statistical analyses of FISH and operational data revealed some correlations, but less than expected. MiDas-DK ([www.midasdk.dk](http://www.midasdk.dk)) will continue the next years and we hope the approach can inspire others to make similar projects in other parts of the world to get a more comprehensive understanding of microbial communities in wastewater engineering.

## **Keywords**

Microbial populations, dynamics, core species, activated sludge, database, nutrient removal, wastewater treatment

## **INTRODUCTION**

Biological wastewater treatment is the largest biotechnological industry in the world and it is essential for protecting human health and the environment. The vast majority of the wastewater treatment plants (WWTP) are based on the activated sludge principle, and an increasing number have biological removal and potential reuse of nitrogen and phosphorus. In order to ensure optimal operation, process optimization and efficient troubleshooting, it is crucial to understand the structure and function of the microbial communities and the factors controlling their composition and activity.

A number of operational problems and difficulties are often encountered in full-scale WWTPs with nutrient removal and despite a steady increasing knowledge about the microbiology and microbial ecology of the WWTP processes, the operation often encounter unsolved problems; such as process instability, foaming and bulking, and insufficient biological N or P removal (Eikelboom, 2000; Jenkins et al., 2004; Seviour and Nielsen, 2010). Solutions to some of these problems are partially understood, but to solve these in a knowledge-driven way, more

fundamental knowledge about the microbial communities is needed. Central questions are the microbial composition, presence of potential core species in similar WWTPs, temporal variations, and factors that regulate the populations, such as wastewater composition, plant design and plant operation. Since 2006 we have gathered such comprehensive information from 50 Danish full-scale WWTP with nutrient removal in order to get a detailed understanding of full-scale plants in a project called “The Microbial Database for Danish Wastewater Treatment Plants with Nutrient Removal (MiDas-DK)”.

Some of the results obtained in MiDas-DK have been published the past 2 years: A conceptual ecosystem model of the enhanced biological phosphorus removal (EBPR) systems (Nielsen et al., 2010; 2011), discovery of several novel polyphosphate accumulating organisms (Nguyen et al., 2011; 2012), discovery of novel glucose fermenters in EBPR plants (Nielsen et al., 2012), a metagenomics analysis of a full-scale EBPR plant (Albertsen et al., 2012), investigation of filamentous *Chloroflexi* in WWTPs (Kragelund et al., 2011) and identity and population dynamics of filamentous bacteria (Mielczarek et al., 2012). Several other papers are recently submitted or in preparation.

We have, however, never given a thorough description of MiDas-DK. The set-up could potentially inspire other researchers to carry out similar investigations other places in the world so results across the world can be obtained and the generic value of the present investigation assessed. In this paper we present the set-up and show how results are collected and stored as well examples of the type of results that have been obtained.

## METHODS

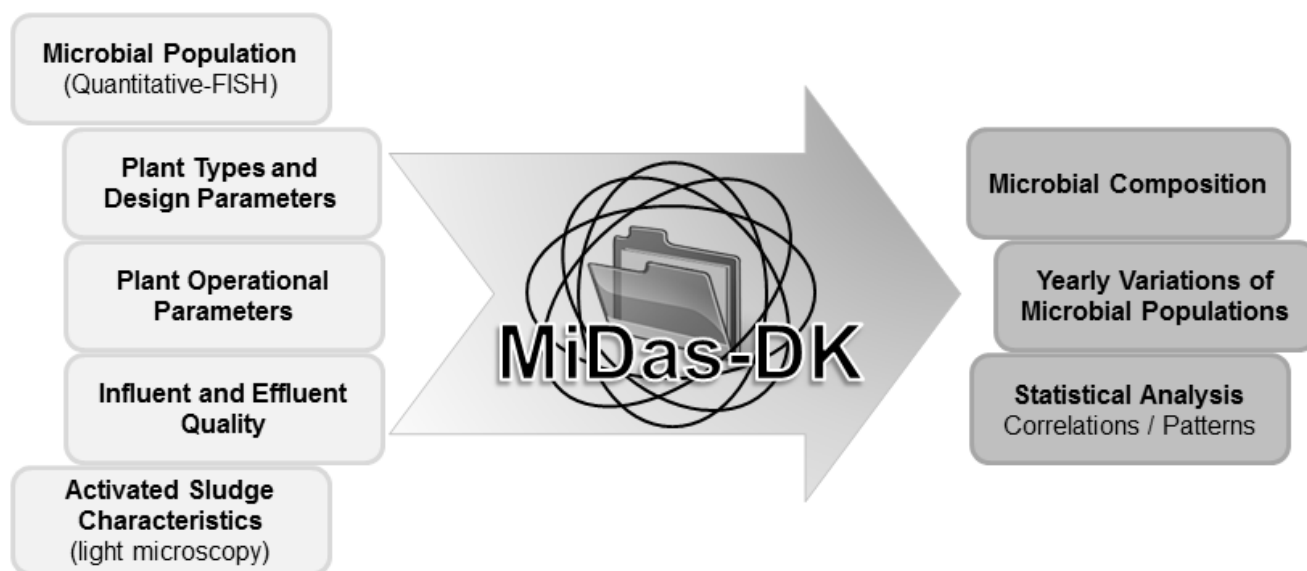
### Treatment plants, gathering of plant info and sampling

More than fifty different full-scale WWTPs have been part of MiDas-DK since 2006. The three first years were primarily a test period to get everything to work regarding sampling, handling, analyses and data interpretation. Twenty-eight full-scale biological nutrient removal plants have been investigated in greater details since 2009.

Extensive information has been (and is) collected on process design, operation, influent and effluent concentrations of specific nutrients, and information on major problems operators have encountered such as bio-P process removal disruptions or foaming/bulking problems. The information is available from all plants since plants operators are very enthusiastic about the project and as all plants are run by local municipalities, all data are easy accessible. Seven plants are sampled four times a year (January, May, August, November) and the remaining 21 plants twice a year (January, August).

Figure 1 shows an overview of the typical workflow of operations in the project. Activated sludge samples are taken from the aerobic process tank and send overnight by postal service to Aalborg University. Each sample is accompanied by a detailed questionnaire gathering all main treatment plant design features and process parameters (monthly averages and data from the day of sampling). At arrival, a fraction of the sample is fixed for fluorescence *in situ* hybridization (FISH) analyses and another frozen at -80°C for future DNA/RNA analysis. Subsequently, within 24 h the samples are visually characterized with the use of light microscopy, according to the protocols by Eikelboom (2000). General sludge properties such as sludge floc size (small,

medium and large), floc structure (open structure, bridging between flocs by filaments, no filament impact), floc properties (strength, form and shape), filament index (FI) (range from 0-5, no filaments – very many filaments) is recorded. Secondly, the bacteria is identified and quantified by quantitative FISH (qFISH) and finally, all the data is gathered and stored in MiDas-DK.



**Figure 1.** Workflow of the Microbial Database MiDas-DK.

### **Identification of microbial community structure with molecular methods**

Quantitative FISH was selected as it provides opportunity to visualize the morphology of the bacteria investigated and quantify their fraction of the entire biovolume as compared to cells stained by the general EUB-probe. A range of approx. 35 oligonucleotide probes targeting different bacterial species was applied (Nielsen et al., 2010; Kragelund et al., 2011; Nguyen et al., 2011). Detailed information about most of these probes is given in probeBase (Loy et al., 2007). The procedure was performed according to the guidelines by Nielsen et al., (2009), and visualised with an epifluorescence microscope. Quantification of probe-defined populations was carried out according to Morgan-Sagastume et al., (2008). Calculated standard deviation is for all values and probes between 16-20% of the average value.

### **16S rRNA gene amplicon sequencing**

The last years also amplicon sequencing (Illumina platform) or pyrosequencing using 454 have been included. 16S rRNA gene PCR products were amplified with the 27F-534R primers and sequenced from the reverse primer (Wu et al., 2010) using the titanium FLX pyrosequencing. Sequences were analysed with QIIME (Caporaso et al., 2010) using the Greengenes database (McDonald et al., 2012), Jan 2011 release. Latest also deep metagenomes have been carried out on selected samples using Illumina HiSeq2000.

## Statistical Approach

In order to reveal potential statistical correlations between probe-defined populations and particular variables of the WWTPs characteristics, e.g. design type, loading etc., a number of different statistical methods were applied. Selected parameters were:

- Design: size, recirculating or alternating operation, side stream hydrolysis, pre-settling, presence of digester
- Operation: MLSS in tanks, pH, DSVI
- Wastewater and load: Incoming COD, industrial load, type of industry, total-N,  $\text{NH}_4\text{-N}$ , total-P and ortho-P, sludge load ( $\text{kgCOD/kgSS/d}$ ), sludge age
- Effluent quality: total N,  $\text{NH}_4\text{-N}$ ,  $\text{NO}_3\text{-N}$ , Total P, ortho-P.

Beside the descriptive statistics we have also applied Principal Component Analysis (PCA), Multivariate analysis of variance (MANOVA), Pearson's correlations and Hierarchical Cluster Analysis and Partial Least Squares (PLS). For correlation and cluster analyses the SPSS 19 package/XLSTAT was used, for PCA analyses the Unscrambler 11.01/XLSTAT

## RESULTS AND DISCUSSION

### Characteristics of Treatment Plants involved

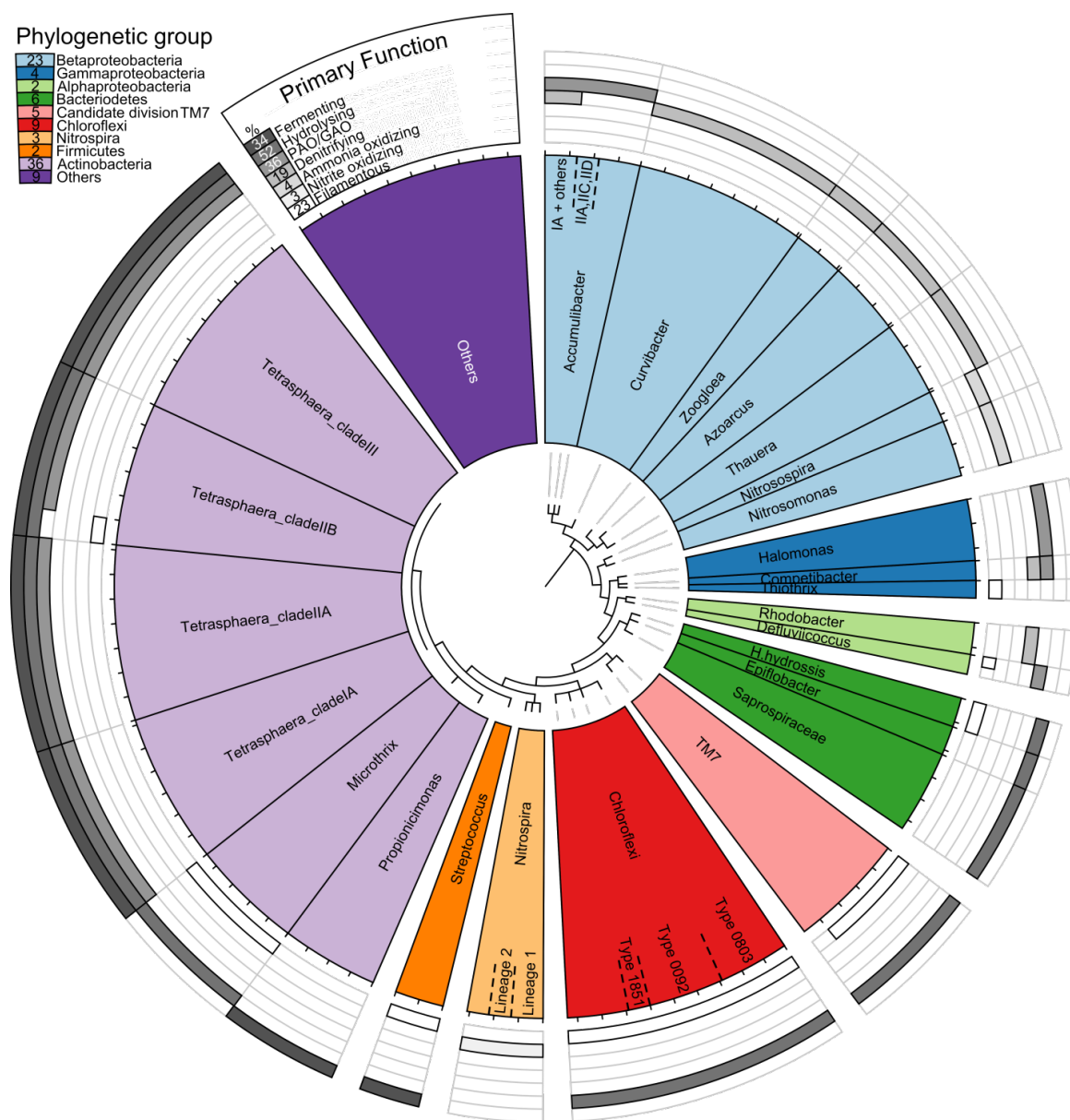
All larger Danish wastewater treatment plants with removal of N and P from municipal wastewater were offered to participate in the MiDas-DK project in 2005. Each plant was asked to pay a fee to be involved which covered a significant part of the project expenses. Fifty plants have participated in one or more years, and 28 plants in all 6 years. Two private companies in the water industry, Krüger A/S and Kemira A/S have also participated. The treatment plants cover a wide range of sizes (from 14,000 – 410,000 PE), and configurations (alternating / recirculating operation). All plants have N-removal (nitrification and denitrification) and majority of them EBPR (anaerobic tank). The industrial load varies between 5% and 74% and come from different industrial sources, e.g. fish and dairy. Some of the plants with EBPR configuration also have a return sludge side-stream hydrolysis (RSS) tank, where 10-25% of the return activated sludge is directed with a retention time of 12-24 h. All plants meet the standard effluent quality of total N below 8 mgN/L and total P below 1 mgP/L. Since all plants pay a fee proportional to the total discharge of N and P, the values are often significantly lower than the standard (typically below 5 and 0.5 mg/L, respectively).

### The Microbial Database as microbial exploration tool

#### Community composition

The microbial composition of activated sludge treatment plants involved in the project was screened to determine the bacterial composition of different functional groups carrying out nitrification, denitrification, polyphosphate accumulation, glycogen accumulation, fermentation, and hydrolysis. The large number of samples from a diverse set of treatment plants allowed us to investigate the microbial communities in great detail and make robust conclusions. In nearly all plants, approx. 80% of the entire biomass could be identified with the probes applied, confirming that they covered most of the diversity present in Danish wastewater treatment plants. Interestingly, the survey showed a large and stable core of identical probe-defined populations present in all plants through the entire study, although with significant variation in abundances.

The core populations is here defined as probe-defined bacteria present in >90% of the plants in an abundance of >0.25%. Figure 2 shows the average abundance of these probe-defined populations based on quantifications in the period 2009-2011. In addition, the known main functional characteristics of these probe-defined populations are summarized.



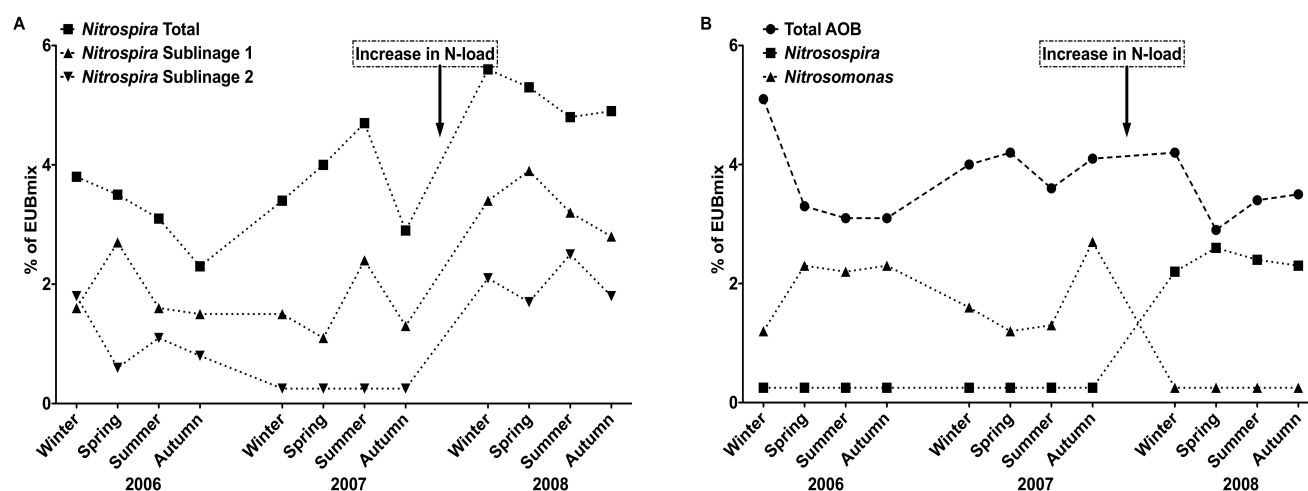
**Figure 2.** The average composition of microbial communities in 25 EBPR plants over 4 years. Most species can be considered as core-species. Their main functions are also included.

As a supplement to qFISH we have conducted amplicon sequencing of 16S rRNA genes from samples in 2011 and 2012. The results confirm the findings by FISH. A more detailed analysis is underway of all samples. However, since the core populations are present in all plants, typical as process-critical species, it is worth the effort to study this limited core community greater detail

in order to understand details of their physiology and ecology. This will lead to better understanding of the processes and lead to development of conceptual and quantitative models to be used for optimization and trouble-shooting.

### Community stability

The long time series have allowed us to look into long-term variations in the different populations. The overall conclusion is that most populations have been surprisingly stable in most plants over the years. Figure 3 shows an example of the nitrite oxidizing *Nitrospira* population dynamics in one of the wastewater treatment plants (Hjørring). It shows the two dominant lineages and 16S rRNA amplicon analysis found the same groups and did not detect any other groups known to be nitrifiers (such as *Nitrobacter*). After one year, a digester was implemented at the plant treating sludge from other small WWTPs, thereby adding a large extra N-load by reject streams. This resulted in increased abundance of *Nitrospira*, which remained constant the following years. Interestingly, there were also a significant change among the ammonium oxidizing bacteria (AOB, *Nitrosospira* and *Nitrosomonas*) at the same time, but we do not know how it was related to the new reject streams.



**Figure 3.** Population dynamics of the nitrifying community in Hjørring WWTP.

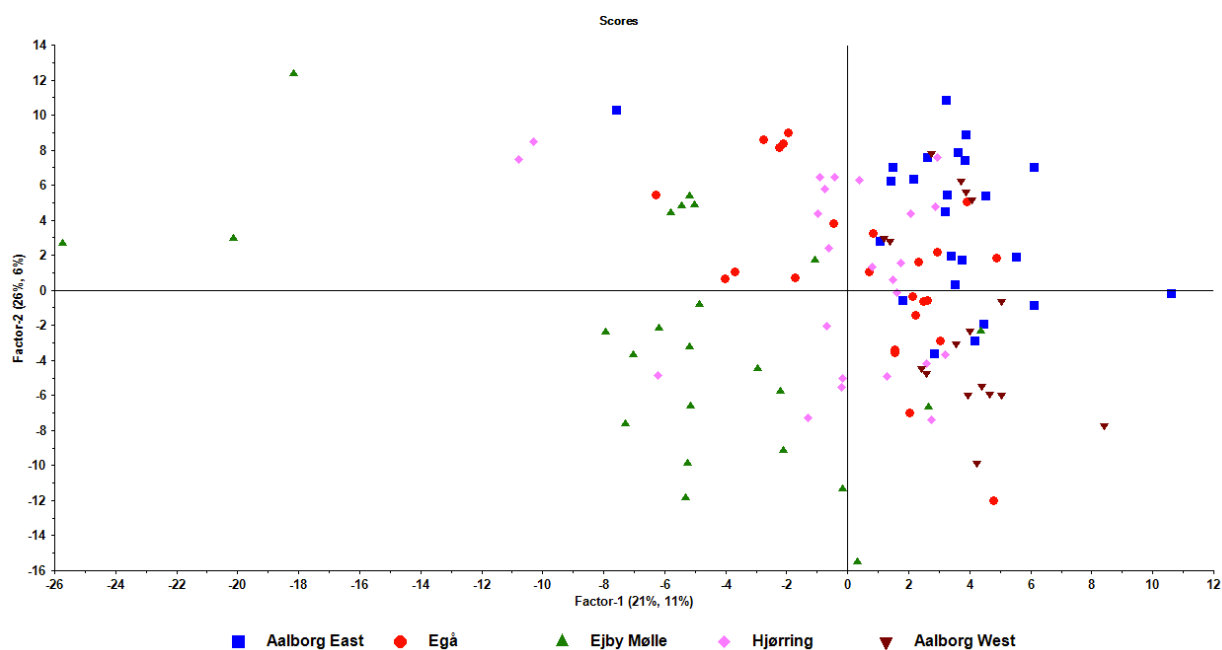
The dominant bacteria involved in EBPR, *Accumulibacter* and *Tetrasphaera*, showed generally a large degree of stability (Mielczarek et al., submitted). Several filamentous bacteria also showed stable abundance (e.g. TM7-related) while others showed larger variations (Mielczarek et al., 2012). This was primarily the probe-defined morphotypes 0803 and 0092 of *Chloroflexi* and to some extent *Microthrix*. The latter showed a clear yearly variation with highest abundance during winter (yearly temperature range 6-20°C). It was difficult to identify a clear yearly variation for other species. An interestingly observation is that the general level of filamentous bacteria varied from year to year. The total abundance was 17% higher in 2009 compared to 2011 (Mielczarek et al., 2012). We have no explanation, but the observation was statistical valid.

The relatively high stability in the Danish plants may be due to the fact that all plants are large nutrient removing plants with long-term operational stability (experienced plant operators with excellent supervision), alternating aerobic/nitrifying and anaerobic/denitrifying conditions, long

sludge age (25-35 days), moderate yearly temperature range (7-20°C), stable pH, and a rather complex wastewater composition. These are all factors that may have a great impact on the temporal stability of the entire community.

#### Each plant is unique

Multivariate analysis of variance (MANOVA) showed that for two functional groups, the filamentous bacteria and bacteria involved in biological phosphorus removal (PAO and GAO), each treatment plant they had their own unique microbial fingerprint over several years, even though their populations seemed very similar between the plants (Mielczarek et al., 2012 and submitted). This is also the case for the complete microbial populations as shown in Fig. 4 with a PLS analysis of the FISH defined populations of five treatment plants investigated over six years. It shows that different treatment plants groups separately. It suggests that the microbial composition of each treatment plant is unique. This observation is important; there seem to be plant-specific factors that control the population and keep the population composition surprisingly constant in each plant over time. However, it is important to stress that the species composition is largely the same, but the relative abundances of the species differ.



**Figure 4.** PLS analysis of microbial populations in 5 full-scale EBPR treatment plants over 6 years.

#### Effect of wastewater and plant design on microbial populations

The exact factors leading to the uniqueness of each plant may be related to wastewater composition or plant design/operation. Various statistical tools were applied to identify these correlations, but it was not possible to find many strong correlations (Table 1 shows few examples). Only one significant strong correlation was found, the sludge loading correlated with *Tetrasphaera* Type 1 (nomenclature after Kong et al., 2005). Also a strong mutual correlation was found between presence of *Saprospiraceae* and *Chloroflexi*. This relation illustrates that *Saprospiraceae* have a specific relationship where they grow attached to the surface of the



filamentous *Chloroflexi*. Other statistical analyses have clustered several filamentous bacteria together as species present in plants with higher industrial load (Mielczarek et al., 2012).

**Table 1.** Examples of correlations found in MiDas-DK

<b>Mutual correlation between bacteria</b>		<b>Correlation strength</b>
<i>Chloroflexi</i> Total	<i>Saprospiraceae</i>	<b>Strong</b>
<i>Thiothrix</i>	<i>H. hydrossis</i>	<b>Medium</b>
<i>Curvibacter</i>	<i>Defluviicoccus</i>	<b>Medium</b>
<b>Correlation between bacteria and process parameter</b>		
<i>Tetrasphaera</i> Type 1	Industrial Contribution	<b>Medium</b>
<i>Tetrasphaera</i> Type 1	Sludge Load	<b>Strong</b>
<i>Accumulibacter</i>	C/P ratio influent	<b>Medium</b>

The few strong correlations in various statistical investigations probably reflect that the plants investigated in MiDas-DK were more similar than expected in respect to both microbial composition and design and operation. This may have influenced the ability to find strong correlations and a larger diversity in plant design and operation may be needed to identify such correlations. However, the long time series of up to 6 years revealed that another strategy may be applicable: to follow specific plants closely over time, preferably together with focus on major changes in wastewater composition or plant design/operation to search for specific factors affecting the different species (as shown in Fig. 3). It was noticed that some correlations and relations found in short-term studies could lead to misinterpretations of the results, since the patterns were not generally valid for different types of plants in longer periods of time.

### **The future of MiDas-DK**

MiDas-DK has proven to be a powerful resource for exploration of the complex activated sludge ecosystems. It has provided a great scientific insight in population structure in a range of different nutrient removal plants. Furthermore, many of the results can be used and has been used to optimize the operation of full-scale plants and carry out trouble-shooting. A continued development to gain new knowledge and transfer this to consultants in the water industry will take place. Many important scientific questions remains to be investigated, such as a better knowledge about the core and non-core populations, specific factors controlling their presence, and establishing a comprehensive (semi) quantitative ecosystem model of the communities.

The MiDas-DK project would not have been possible without the strong collaboration with the treatment plants involved. One or two yearly workshops for all participating treatment plants have taken place and they have been very important to inform about important results and give guidance about how these can be transferred to operational guidelines. Furthermore, publications of small Danish articles and participation in courses and workshops for plant operators and consultants from the wastewater industry have been invaluable and these activities will continue.

Among the new challenges is to investigate whether the community structure investigated in the Danish plants are similar to communities in other climates and cultures worldwide. Similar projects in other countries are needed and the concept, and experience obtained through MiDas-DK could be a useful model for others.

Since 2011 we have also established a database for the study of the microbial communities in biogas reactors (primarily digesters on WWTP). There is currently a major expansion of biogas reactors in Denmark and abroad due to a growing awareness of energy production from wastewater treatment plants, manure and other wastes. Operational stability of these reactors is not always high, and problems can probably often be attributed to microbiological conditions. We anticipate that the results can help solving many of these problems and help optimizing the operation.

## CONCLUSION

- The construction of the database over Danish treatment plants (MiDas-DK) has greatly contributed in obtaining new insight into the most important microbial populations in a range of different Danish full-scale treatment plants with nutrient removal.
- The microbial communities of all investigated EBPR plants were very similar, revealing over a period of 6 years a core of approx. 30 probe-defined species that were typical for all Danish plants with nutrient removal.
- The main controlling factors affecting the activated sludge microbial populations are likely substrate composition of the inflowing wastewater and the major operational changes in the treatment plant.
- The approach used in MiDas-DK is now extended also to digesters and has the potential to be used worldwide in a variety of additional water engineering systems with “similar” microbial populations.

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